

## CLAIMS

1. A sulfur atom-free enzyme protein comprising 18 types of L-amino acid residue: L-alanine; L-aspartic acid; L-glutamic acid; L-phenylalanine; L-glycine; L-histidine; L-isoleucine; L-lysine; L-leucine; L-asparagine; L-proline; L-glutamine; L-arginine; L-serine; L-threonine; L-valine; L-tyrosine; and L-tryptophan.
2. The sulfur atom-free enzyme protein according to claim 1 which retains the activity of the original enzyme protein and has oxidation resistance, wherein L-cystein and L-methionine residues in enzyme proteins comprising 20 types of L-amino acid residue: L-alanine; L-aspartic acid; L-glutamic acid; L-phenylalanine; L-glycine; L-histidine; L-isoleucine; L-lysine; L-leucine; L-asparagine; L-proline; L-glutamine; L-arginine; L-serine; L-threonine; L-valine; L-tyrosine; L-tryptophan; L-cystein; and L-methionine, are substituted with 18 types of L-amino acid residue: L-alanine; L-aspartic acid; L-glutamic acid; L-phenylalanine; L-glycine; L-histidine; L-isoleucine; L-lysine; L-leucine; L-asparagine; L-proline; L-glutamine; L-arginine; L-serine; L-threonine; L-valine; L-tyrosine; and L-tryptophan.
3. The sulfur atom-free enzyme protein according to claim 2 wherein amino acid substitution is carried out by site-directed mutagenesis using synthetic DNA.
4. The sulfur atom-free enzyme protein according to any one of claims 1 to 3 wherein the enzyme activity is oxidation-reduction activity or hydrolysis activity.
5. The sulfur atom-free enzyme protein according to any one of claims 1 to 4, which retains the activity of dihydrofolate reductase and has oxidation resistance.
6. The sulfur atom-free enzyme protein according to any one of claims 1 to 4, which retains the activity of xylanase and has oxidation resistance.
7. A method of producing a sulfur atom-free enzyme protein prepared by combined mutation comprising the following steps:
  - (1) preparing a mutant gene by substituting an initiation codon encoding L-methionine in a DNA sequence encoding an enzyme protein consisting of a total length of m amino acids, and comprising n number of sulfur atom-containing amino acids (sulfur-containing amino acids), wherein a position of a sulfur-containing amino acid on the sequence is  $A_i$  ( $i = 1$  to  $n$ ), by L-methionine-L-alanine, L-methionine-L-serine, or L-methionine-L-proline codon, expressing the prepared mutant gene in a host cell, measuring enzyme activity of the obtained mutant enzyme protein, and selecting the protein with the highest activity, thereby obtaining a substitution mutant A1/MA1;

- (2) preparing a mutant gene in which codons encoding sulfur-containing amino acids at another sites  $A_i$  ( $i = 2$  to  $n$ ) are substituted with codons encoding another amino acids among the 18 types of amino acid according to claim 1, expressing the prepared mutant gene in a host cell, measuring enzyme activity of the obtained mutant enzyme protein, and selecting  $p$  number of mutant enzyme proteins having enzyme activity, thereby obtaining substitution mutants  $A_i/B_{ij}$  ( $j = 1$  to  $p$ );
- (3) selecting a maximum of 3 substitution mutants having the highest activity  $A_i/B_{i1}$ ,  $A_i/B_{i2}$ , and  $A_i/B_{i3}$ , wherein activity decreases in order,  $A_i/B_{i1} > A_i/B_{i2} > A_i/B_{i3} > \dots > A_i/B_{ip}$ ; and
- (4) selecting substitution mutants having activity in respect of sulfur-containing amino acids at all sites  $A_i$  ( $i = 2$  to  $n$ ) in the same manner as (2) and (3) above, preparing a maximum of  $3 \times (n-1)$  mutants being all combinations of these mutants with the mutant of  $A_1/MA_1$ , measuring the enzyme activity thereof, and obtaining a mutant enzyme protein having activity greater than or equivalent to the original enzyme protein.

8. A method of producing a sulfur atom-free enzyme protein prepared by stepwise mutation comprising the following steps:

- (1) preparing a mutant gene by substituting an initiation codon encoding L-methionine in a DNA sequence encoding an enzyme protein consisting of a total length of  $m$  amino acids, and comprising  $n$  number of sulfur atom-containing amino acids (sulfur-containing amino acids), wherein a position of a sulfur-containing amino acid on the sequence is  $A_i$  ( $i = 1$  to  $n$ ), by L-methionine-L-alanine, L-methionine-L-serine, or L-methionine-L-proline codon, expressing the prepared mutant gene in a host cell, measuring enzyme activity of the obtained mutant enzyme protein, and selecting the protein with the highest activity, thereby obtaining a substitution mutant  $A_1/MA_1$ ;
- (2) preparing a mutant gene in which codons encoding sulfur-containing amino acids at  $A_2$  of  $A_1/MA_1$  mutant is substituted with a codon encoding another amino acid being one of the 18 types of amino acid according to claim 1, expressing the prepared mutant gene in a host cell, measuring enzyme activity of the obtained double mutant enzyme protein, and selecting a maximum of 3 triple mutants with the highest activity;
- (3) preparing a mutant gene in which a codon encoding a sulfur-containing amino acid at each  $A_3$  of the obtained double mutants is substituted with a

codon encoding another amino acid being one of the 18 types of amino acid according to claim 1, expressing the prepared mutant gene in a host cell, measuring enzyme activity of the obtained triple mutant enzyme protein, and selecting a maximum of 3 triple mutants with the highest activity; and

- (4) in the same manner as described above, preparing a quadruple mutant, ..., multiple number of n mutant, inspecting enzyme activity of the last multiple number of n mutant, and preparing a mutant enzyme protein having activity greater than or equivalent to that of the original enzyme.

9. The process for producing a sulfur atom-free enzyme protein prepared by the stepwise mutation according to claim 8 wherein the order of stepwise mutation sites is according to any one of (n! types) permutations and combinations of A1, A2, ..., An.

10. A process for producing a sulfur atom-free enzyme protein wherein, in an enzyme protein consisting of a total length of m amino acids and comprising n number of sulfur-containing amino acids, in which a site of a sulfur-containing amino acid on the sequence is  $A_i$  ( $i = 1$  to  $n$ ), a process according to claim 7 is adopted at k number of sites and a process according to claim 9 is adopted at remaining  $n - k$  number of sites.